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# Comparison of plantain plantlets propagated in temporary immersion bioreactors and gelled medium during *in vitro* growth and acclimatization

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**Abstract**

The current work compared the physiological characteristics of plantain (*Musa* AAB) plantlets micropropagated in temporary immersion bioreactors (TIB) and on a gelled medium (GM). The plantlets were evaluated during *in vitro* growth (in the shoot elongation phase) and at the end of *ex vitro* acclimatization. TIB improved rooting and gave rise to longer shoots and higher dry mass. Respiration rate was the highest at the beginning of shoot elongation in both the TIB and GM plantlets. Photosynthetic rate in TIB was significantly higher than in GM from the midpoint of acclimatization, whereas a pyruvate kinase (PK) activity was lower. Starch accumulation was *ca.* two fold higher in corms than in leaves and always higher in the TIB than GM plantlets. The higher expression of genes coding for carbon metabolism enzymes PK and phospho*enol*pyruvate carboxylase (PEPC) in TIB than in PM indicates a more important role of an autotrophic metabolism in the TIB plantlets when compared to the GM ones. The accumulated reserves were used during the first days of acclimatization leading to the higher survival rates and to the better plant quality of the TIB plantlets.

*Additional key words:* acclimatization, ethylene, *Musa* spp., PEPC, peroxiredoxins, photosynthesis.

## Introduction

Banana and plantain are included in the genus *Musa* and most of their agronomic and physiologic properties are similar. Plantain is grown almost exclusively by small-scale-subsistence farmers and plays an important socioeconomic role in many developing countries of the tropics and subtropics (Panis and Thinh 2001). Banana and plantain are usually propagated from suckers and bits (pieces of corm with attached growing points), but export-type commercial companies more commonly use tissue-cultured plantlets free from sucker borne diseases and pests. The interest in the use of liquid media in micropropagation (Ziv 2005), and of temporary immersion bioreactors (TIB) in particular, has increased during the last decade. On average, TIB guarantees better plant quality and higher multiplication rates (Escalona *et al*. 1999, Lorenzo *et al.* 2001, Etienne and Berthouly 2002, Aragón *et al.* 2010). However, conventional micropropagation systems using gelled medium (GM) are still widely used for a high number of crops. Both systems comprise specific aspects that allow good plant growth and favour acclimatization although environmental conditions associated to *in vitro* growth in either GM or TIB confer specific characteristics to the plantlets which are directly responsible for their *ex vitro* performance. TIB propagation takes place in automated systems that promote the aeration of containers. This ventilation of the headspace is paramount to remove volatile compounds such as ethylene (Roels *et al.* 2006) and to promote the recirculation of carbon dioxide necessary for photosynthesis further enhancing autotrophic carbon metabolism in leaves. The metabolic and physiological behavior of TIB-produced plantlets has not yet been studied in depth (Teisson and Alvard 1995, Etienne and Berthouly 2002, Escalona *et al*. 2003), although it is now known that TIB plantain plantlets cope better with reactive oxygen species (ROS) produced at

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*Abbreviations*: BAP - 6-benzylaminopurine; GM - gelled medium; PEPC - phospho*enol*pyruvate carboxylase; PK - pyruvate kinase; PPFD - photosynthetic photon flux density; Prxs - peroxiredoxins; SS - starch synthase; ROS - reactive oxygen species; TIB - temporary immersion bioreactors.
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the transfer from *in vitro* to *ex vitro* conditions (Aragón *et al*. 2010).

Once the plantlets are ready for shifting from *in vitro* conditions, they must be acclimatized to adapt to greenhouse and later to field (Ma and Shii 1974, Martín *et al*. 2009). During this hardening period, plantlets undergo physiological adaptations to external factors like water availability, temperature, air humidity, and nutrient supply. Starch accumulation in leaves of *in vitro* plantlets is vital for the acclimation to *ex vitro* conditions (Capellades *et al.* 1991, Cayón 2001, Bello-Pérez *et al.* 2002) and leaves play an important role as carbon source during the transition to autotrophy. Also, particular roles were reported for phospho*enol*pyruvate carboxylase (PEPC) and pyruvate kinase (PK) during acclimatization of TIB propagated plantlets, PEPC being responsible for the mobilization of sugars through an anaplerotic route to guarantee the supply of carbon skeletons for amino acid synthesis during *in vitro* propagation (Aragón *et al.* 2005). Plantain propagation in TIB was also studied under $CO_2$ enrichment (Aragón *et al*. 2009) that increased overall plant quality and led to a favourable balance between the starch content in leaves and corms.

The mechanisms by which TIB grown plantlets are able to better sustain and overcome oxidative stress than GM grown plantlets, thus enhancing their propagation capacity and improving their growth, were recently elucidated including the partial characterization and the description of the role of peroxiredoxins (Prxs) in this transition (Aragón *et al*. 2010). In the current study, *in vitro* growth of plantain plantlets in TIB and GM is characterized according to ethylene accumulation in the headspace, photosynthetic rate, stomatal conductance, starch accumulation, and PK and PEPC activities. The expression of genes coding these enzymes was monitored during the acclimatization of those plantlets. Finally, gene expression and protein profile of Prx Q, E, and F were analyzed at the end of acclimatization.

## Materials and methods

**Plants and *in vitro* culture:** Plantain shoots (*Musa* AAB cv. Cemsa 3/4) were micropropagated in a gelled medium (GM) containing Murashige and Skoog (1962; MS) salts and vitamins (MS), 30 g $dm^{-3}$ sucrose, 13.3 µM 6-benzylaminopurine (BAP), and 2.5 g $dm^{-3}$ *Gelrite* for 3 subculture cycles of 28 d. After this, only shoots with a corm diameter of at least 3 mm and 3 cm length were used for the elongation phase lasting 21 d. Two different techniques, GM and temporary immersion bioreactors (TIB), were used. In both methods, the basal medium was MS supplemented with 30 g $dm^{-3}$ sucrose, without plant growth regulators, with pH adjusted to 5.8 before autoclaving at 121 ºC and 118 kPa for 20 min. GM plantlets were placed in glass vessels of 500 $cm^3$ total volume containing 150 $cm^3$ of media, jellified with 2.5 g $dm^{-3}$ *Gelrite*, 5 shoots per vessel. TIB was performed in pairs of the same vessels, one vessel containing 5 shoots and the other 150 $cm^3$ of a liquid medium (Escalona *et al.* 1999). An automatic pump system allowed the forced ventilation of the TIB system with the immersion of the shoots in the media for 4 min every 3 h and the subsequent renewal of the headspace. Air pressure from an air compressor pushed the medium from one container to the other to immerse the plants completely. The airflow was reversed to withdraw the medium from the culture container. Atmospheric air with a $CO_2$ concentration of 375 µmol $mol^{-1}$ was used in TIB, and in GM, the $CO_2$ concentration was variable depending on the plant metabolism, as it is a closed system. Cultures were maintained at temperature of 25 ºC, a 16-h photoperiod, and a photosynthetic photon flux density (PPFD) of 45 ± 5 µmol $m^{-2}$ $s^{-1}$ (cool-white fluorescent lamps *Daylight F40T12/D*, 40 W, *Sylvania*, Danvers, USA). The plantlets were harvested weekly during elongation (E0, E7, E14, and E21) for ethylene and morphological and physiological parameter determinations. At each time point, the following growth parameters were evaluated in 15 plantlets: a shoot length, a diameter of the base of the pseudostem, a number of leaves per shoot, a length and maximum width of the main leaf, a leaf area (LA calculated by the approximation to the ellipse area; Nakamura *et al*. 2005), a number of roots per shoot, a fresh mass and a dry mass (after drying the whole plants at 50 ºC until a constant mass).

***Ex vitro* acclimatization:** After elongation, the shoots produced in GM and TIB were transplanted to pots containing a sterilized mixture of water saturated peat and *Perlite* (1:1, v/v) and placed in glass chambers (450 $dm^3$; *500E*, *Aralab*, Porto Salvo, Portugal). PPFD was 200 ± 10 µmol $m^{-2}$ $s^{-1}$ and a 16-h photoperiod. The initial relative humidity (RH) was set at 98 % and gradually decreased until it reached the ambient value. Temperature was 25 ± 2 ºC during the day and 22 ± 1 ºC during the dark period. The plants were sampled after 0, 3, 7, and 14 d of acclimatization (A0, A3, A7, and A14) for the expression of genes coding PK, PEPC, and SS. At the end of acclimatization (A21), the peroxiredoxin gene expression was quantified. One time point was coincidental between elongation and acclimatization (E21 = A0) and the whole experiment lasted 42 d (21 + 21).

**Quantification of ethylene in the headspaces:** An ethylene concentration in the headspaces was determined by gas chromatography. Samples of the headspace atmosphere were taken with a syringe and needle perforating the silicone tube connecting the vessel pairs in the TIB systems or protruding from the GM vessels. The samples were injected in sealed rubber cap vials until total atmosphere was renovated. For each time point, at least three independent samples (1 $cm^3$) were analyzed in a gas chromatograph (*PYE Unicam 204*, Cambridge, UK)

equipped with a flame ionization detector (FID) and a *Porapak Q* column (80 - 100 mesh, 1.5 m length, and 4 mm diameter). Nitrogen was used as carrier gas at a flow rate of 30 $cm^3$ $min^{-1}$, and the injector, column, and detector temperatures were 25, 100, and 150 ºC, respectively. An external ethylene standard (29 $mm^3$ $dm^{-3}$) was used as reference.

**Photosynthetic rate, stomatal conductance, and transpiration rate:** During the elongation phase, fully expanded leaves were sampled weekly in the middle of the photoperiod. The gas exchange parameters were recorded with a portable *CIRAS-2* photosynthesis system (*PP Systems*, Herts, UK). The leaf used occupied the whole area of the cuvette (*PLC6*; 2.5 $cm^2$). The $CO_2$ concentration and the humidity of the air entering the leaf chamber were 375 µmol $mol^{-1}$ and 80 %, respectively, PPFD was 600 µmol $m^{-2}$ $s^{-1}$ (maximum at which photosynthesis was stable), and an ambient temperature of 25 °C. Measurements of a photosynthetic rate, stomatal conductance, and transpiration rate were performed on leaves of three plantlets, with 10 measurements per plantlet ($n$ = 30).

**Starch quantification:** Starch content of the corms and leaves was determined in the *in vitro* and *ex vitro* grown plantlets. One gram of leaf material was frozen in liquid nitrogen and ground with a mortar and a pestle. Soluble sugars were removed with 80 % (v/v) ethanol. The extract was centrifuged at 10 000 *g* and 4 ºC for 20 min and the pellet was suspended in 5 $cm^3$ of 0.2 M KOH. After the alkaline hydrolyses, an enzymatic treatment with β-amyloglucosidase (EC 3.2.1.3, *Sigma-Aldrich*, St. Louis, USA) was applied for total starch degradation (Thomas *et al.* 1983). Soluble sugars derived from the starch degradation were measured by the anthrone method (Van Handel 1968) using a spectrophotometer *Ultrospec II*, (*GE Healthcare*, Little Chalfont, UK) at 620 nm using potato starch (*Sigma-Aldrich*) as standard.

**Protein extraction and enzyme activity assays:** Leaves (0,25 g) were collected in the middle of the photoperiod and immediately frozen in liquid nitrogen. The frozen samples were ground with a mortar and a pestle and the resulting powdered plant material was resuspended in 1 $cm^3$ of a 50 mM Hepes-KOH buffer containing 12 mM $MgCl_2$, 1 mM ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*-tetraacetic acid (EGTA), 1 mM ethylenediamine-tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 10 % (v/v) glycerol, 2 mM benzamidine, and 2 mM amino-*n*-caproic acid, pH 7.4 according to Siegel and Stitt (1990). The extract was filtered through *Miracloth* and centrifuged at 15 000 *g* and 4 ºC for 20 min. Supernatants were desalted through *PD-10* columns (*GE-Healthcare Life Sciences*, Buckinghamshire, UK) and used for all the determinations. Protein was quantified by the method of Bradford (1976).

Phospho*enol*pyruvate carboxylase (PEPC; EC 4.1.1.31) was immediately measured spectrophotometrically by coupling the reaction to NADH oxidation mediated by malate dehydrogenase. The standard assay solution contained 50 mM Tris-HCl (pH 7.6) complemented with 20 µM $NaHCO_3$, 130 nM NADH, 10 µM $MgCl_2$, 5 µM DTT, 1 U malate dehydrogenase (MDH) (EC 1.1.1.37) (*Sigma-Aldrich*), 1 mM glucose-6-phospate, 50 $mm^3$ of the desalted enzyme solution in a final volume of 1 $cm^3$. Reactions were initiated by addition of 3.25 µM phospho*enol*pyruvate (adapted from Geigenberger and Stitt 1991). The reaction was assayed at 25 ºC by monitoring the consumption of NADH at 340 nm using the spectrophotometer *Ultrospec II* (Hdider and Desjardins 1994).

Pyruvate kinase (PK; EC 2.7.1.40) was assayed in a reaction coupled with the lactate dehydrogenase (LDH, EC 1.1.1.37) (*Sigma-Aldrich*) reaction at 25 ºC by monitoring NADH consumption at 340 nm. The assay solution contained 50 mM imidazole-HCl complemented with 2 mM PEP, 2 mM ADP, 10 mM $MgCl_2$, 30 mM KCl, 0.15 mM NADH, and 2.5 U $cm^{-3}$ of desalted rabbit muscle LDH (EC 1.1.1.27) (*Sigma-Aldrich*) in a final volume of 1 $cm^3$ at pH 7.0 (Lin *et al.* 1989). Both activities were expressed in units, with 1 U = 1 µmol NADH oxidized per min. Three readings of each sample were performed.

**Protein extraction and two-dimensional electrophoresis (2-DE):** Frozen leaf material (0.5 g) previously collected at day 21 of acclimatization in the middle of photoperiod was ground in the presence of liquid nitrogen. Proteins were precipitated at -20 °C for 1 h with acetone containing 10 % (m/v) trichloroacetic acid (TCA) and 60 mM DTT, and centrifuged at 15 000 *g* and at 4 °C for 15 min. The resulting pellet was washed with acetone containing 60 mM DTT at -20 °C for 1 h and centrifuged again. This pellet was dried under vacuum and used as crude extract after being re-dissolved at 25 °C for 2 h in a buffer containing 7 M urea, 2 M thiourea, 0.4 % (v/v) *Triton X-100*, 4 % (m/v) 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate (CHAPS), 60 mM DTT, and a 1 % (v/v) immobilized pH gradient buffer (IPG; *GE Healthcare Life Sciences*). Protein was quantified by the method of Bradford (1976), modified by Ramagli (1999). The samples were incubated at 25 °C for 1 h in a re-hydration buffer containing 8 M urea, 4 % (m/v) CHAPS, 60 mM DTT, and 1 % (v/v) IPG buffer.

Isoelectric focusing (IEF) was carried out using *Ready-Strip-IPG-Strips* (*Bio-Rad*, Hercules, USA) with linear pH gradient 3 - 10 and 40 mg of each protein sample were loaded. The IEF was carried out using a *Protean i12 IEF* cell (*Bio-Rad*) with rehydration at 50 V for 12 h, followed by four consecutive steps in the following conditions: 250 V $h^{-1}$, 500 V $h^{-1}$, 8000 V for 2.30 h, and 8000 V $h^{-1}$ until reaching 30 000 V. Then the IEF strips were equilibrated for 15 min in 50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30 % (v/v) glycerol, 1 % (m/v) SDS, and 65 mM DTT. To remove DTT excess, strips were equilibrated in the same buffer for

15 min replacing DTT with 135 mM iodoacetamide. SDS-PAGE in the second dimension was then performed in denaturing polyacrylamide gels according to the procedure of Laemmli (1970) without stacking gel.

**Immunoblotting:** Polyclonal antibodies against chloroplast-located type II Prx E, chloroplast-located Prx Q, and mitochondria-located type II Prx F were kindly supplied by Professor K-J Dietz (Bielefeld University, Bielefeld, Germany). A Western blot analysis was performed after 2-DE through the transfer of proteins to a nitrocellulose membrane (*Millipore*). The membranes were probed with the respective antibodies using the procedure described by Ferreira *et al*. (1996) and staining was performed with an *AP* conjugate substrate kit (*Bio-Rad*).

**RNA isolation and cDNA preparation:** Total RNA from frozen leaf material collected along the acclimatization phase (A0, A3, A7, and A14) was extracted by adapting the method of Chang *et al*. (1993). An extraction buffer consisted of cetyltrimethyl-amonnium bromide (CTAB; 2 %, m/v) complemented with polyvinylpolypyrrolidone (PVPP; 2 %, m/v), 100 mM Trizma-HCl, 25 mM $Na_2EDTA$, and 2 M NaCl, pH 8.0, heated to 85 ºC prior to the addition of 400 $mm^3$ of 2-mercaptoethanol. Tissues were ground to powder in liquid $N_2$ and 20 $cm^3$ of the extraction buffer was added. The same volume of chloroform:isoamyl alcohol 24:1 was then added. This step was followed by centrifugation at 12 000 *g* and 20 ºC for 30 min and repeated once. The aqueous phase was transferred to a new tube, and 0.25 volumes of 10 M LiCl was added. The sample was incubated at 0 ºC overnight and after centrifugation at 12 000 *g* and 4 ºC for 20 min, the pellet was recovered. A 1.5 $cm^3$ volume of buffer (10 mM Trizma-HCl, 1 mM $Na_2EDTA$, 1 M NaCl, and 0.5 %, m/v, sodiumdodecyl sulphate, SDS, pH 8.0, previously heated to 37 ºC) was added. Ethanol (100 %, 2.5 volumes) was added and the samples were incubated at -80 ºC for 1 h and then washed with 70 % (v/v) ethanol. After drying, the RNA was resuspended in the desired volume of distilled water.

RNA samples were treated with *RQ1* RNase-free DNase (*Promega*, Madison, WI, USA). cDNA was synthesized from 2 µg of total RNA using oligo (dT) in a 20 $mm^3$ reaction volume with revert aid reverse trans-criptase (*Fermentas Life Science*, Helsingborg, Sweden) according to the manufacturer's recommendations.

**Real time PCR:** Primer pairs used for amplification of the genes studied are presented in Table 1. The genomic sequences for plantain available in the GeneBank database (http://www.ncbi.nlm.nih.gov/genbank/) were used and when plantain sequences were not available, the respective orthologs of *Vitis vinifera*, previously tested by our group, were used.

A 20 $mm^3$ volume of the reaction mixture was composed of cDNA, 0.5 µM gene-specific primers and a master mix *iQ EVA Green Supermix* (*Bio-Rad*) and iQ5 real time PCR (*Bio-Rad*) was used. Amplification of PCR products was monitored *via* intercalation of *EVA Green* (included in the master mix). The following program was applied: initial polymerase activation at 95 ºC for 3 min, then 40 cycles at 95 ºC for 15 s (denaturation), at 57 ºC for 30 s (annealing), and at 72 ºC for 20 s (extension) with single fluorescence reading taken at the end of each cycle. Each run was completed with a melting curve analysis to confirm the specificity of amplification and the lack of primer dimers. Further, PCR products were resolved on 2 % (m/v) agarose gels run at 4 V $cm^{-1}$ in a Tris-acetate-EDTA buffer (TAE) together with a 50-bp DNA-standard ladder (*Invitrogen*) to confirm the presence of a single product of the desired length.

To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔRn) versus a cycle number, baseline data were collected between the cycles 5 and 17. All amplification plots were analyzed with an Rn threshold of 0.3 to obtain Cq (quantification cycle) and the data obtained were exported into a *MS Excel* workbook. In order to compare data from different PCR runs or cDNA samples, Cq values were normalized to the Cq value of *Act2*, a housekeeping gene expressed at a relatively high and constant level (Coito *et al*. 2012) and the ΔΔCq method was used for the quantification of gene expression.

**Statistical analysis of the results:** Each experiment was performed twice and the combined results of both experiments were analyzed. The number of replicates performed is indicated in the legends of figures and tables. Statistical analyses were carried out using *SPSS v. 12* (Pérez 2005) and the treatments were compared using the non-parametric analysis by Kruskall-Wallis, Dunnett, or Mann Whitney tests at 5 % probability.

## Results

In the present study, plantain plantlets propagated in the TIB and GM conditions were compared during *in vitro* growth and during acclimatization.

The ethylene concentration in the headspace of the GM and TIB systems was analyzed (Table 2). In GM, an increase began at E14 and reached 0.11 $mm^3\ dm^{-3}$ in the last week. Conversely, in the headspace of the TIB cultures, ethylene was only detected at E21 and its concentration was significantly lower (0.02 $mm^3\ dm^{-3}$).

At the end of the elongation phase, the TIB propagated plantlets had significantly longer shoots and main leaves, a higher number of leaves and roots, and higher fresh and dry masses when compared to the GM grown plantlets (Table 2). In the TIB cultures, a high rate of plant growth during the first weeks often occurred, slowing down towards the end when the stem diameter

Table 1. RT-qPCR primers used for gene expression (sequences from plantain or from grape vine).

| Protein | Acc. number | Primer | Sequence |
|---|---|---|---|
| Actin | AF369525.1 | ACT-F | 5'-TGGATTCTGGTGATGGTGTGAGTC-3' |
| *Vitis* | | ACT-R | 3´-CAATTTCCCGTTCAGCAGTAGTGG-5´ |
| Pyruvate kinase 1 | Mu11M06_14 | PK1-F | 5´-TTCACAACACACCTGGAGAACC-3´ |
| *Musa* | | PK1-R | 5'-GATGTCACCTCCTCCTCGTCTG-3' |
| Phospho*enol*pyruvate carboxylase | Z99987.1 | PEPC-F | 5´-GGTAGTGGAAATGTCTCGCTTGG-3´ |
| *Musa* | | PEPC-R | 5´-GGCTTCTCAGGTTCATGGATTGC-3´ |
| Starch synthase | JQ861709 | SS-F | 5´-AATCTCACGGGATATACAAGAATGC-3´ |
| *Musa* | | SS-R | 5´-GCCTGTTAGACCCATCAGTGAAATC-3´ |
| Type II peroxiredoxin_E | JN392723 | PrxE-F | 5'-AATCTACCATAGGAATGCTCGTTGC-3' |
| *Vitis* | | PrxE-R | 3´-AATCAGACACAGGAAACCACAAACC-5´ |
| Type II peroxiredoxin_F | JN392724 | PrxF-F | 5'-CGAAGCATGATGATGAAATCAACGG-3' |
| *Vitis* | | PrxF-R | 3´-GCACCAGAAACCTTAACCTCGGATG-5´ |
| Peroxiredoxin Q | JN392725 | PrxQ-F | 5'-ACCTTCCTCACTCTTAATGGCTTCC-3' |
| *Vitis* | | PrxQ-R | 3´-CTTTCCTCACCTTGTTTCCGTCATC-5´ |

Table 2. Effect of the micropropagation system (GM or TIB) on plantain ethylene content in headspace ($n$ = 3), morphology ($n$ = 15), photosynthetic rate, stomatal conductance, and transpiration rate ($n$ = 30), starch content in leaves and corms, and activity of pyruvate kinase (PK) and posphoenolpyruvate carboxylase (PEPC) ($n$ = 9) during *in vitro* elongation measured at days 0, 7, 14, and 21. Values followed by different letters within each row are significantly different at 5 % level. One unit (U) corresponds to 1 µmol of substrate processed per hour. * - Vestigial concentrations of ethylene measured, impossible to quantify.

| Parameters | E0 | E7 | | E14 | | E21 | |
|---|---|---|---|---|---|---|---|
| | | GM | TIB | GM | TIB | GM | TIB |
| Ethylene [$mm^3\ dm^{-3}$] | control | * | * | 0.024 b | * | 0.112 a | 0.019 b |
| Shoot length [cm] | 3.02 c | 3.23 c | 3.17 c | 3.63 b | 4.19 a | 3.85 b | 4.18 a |
| Stem diameter [cm] | 0.41 c | 0.51 b | 0.53 b | 0.57 b | 0.56 b | 0.73 a | 0.71 a |
| Number of leaves per shoot | 1.40 c | 2.07 b | 2.27 b | 2.33 b | 2.73 a | 2.07 b | 2.80 a |
| Length of main leaf [cm] | 1.49 c | 2.09 b | 1.75 c | 2.18 b | 2.57 a | 2.04 b | 2.46 a |
| Width of main leaf [cm] | 0.86 b | 1.06 ab | 0.79 b | 1.21 a | 0.93 b | 1.15 a | 0.97 b |
| Leaf area [$cm^2$] | 1.28 b | 2.21 ab | 1.38 b | 2.63 a | 2.39 a | 2.34 a | 2.38 a |
| Number of roots per shoot | 0.00 c | 0.00 c | 0.00 c | 0.00 c | 1.80 b | 1.60 b | 2.93 a |
| Fresh mass [g] | 0.67 b | 0.60 b | 0.49 c | 0.71 b | 0.86 a | 0.77 b | 0.86 a |
| Dry mass [g] | 0.03 b | 0.03 b | 0.03 c | 0.04 b | 0.04 a | 0.04 b | 0.04 a |
| Photosynthetic rate [$\mu mol(CO_2)\ m^{-2}\ s^{-1}$] | -7.63 c | -10.92 c | -12.59 c | 1.92 b | 8.86 a | 2.12 b | 6.04 a |
| Stomata conductance [$mol(H_2O)\ m^{-2}\ s^{-1}$] | 0.16 b | 0.17 b | 0.15 b | 0.33 a | 0.05 c | 0.39 a | 0.06 c |
| Transpiration rate [$mmol(H_2O)\ m^{-2}\ s^{-1}$] | 1.33 b | 0.81 c | 2.17 a | 1.34 b | 1.56 b | 1.29 b | 1.90 a |
| Starch, leaves [$g\ g^{-1}$(d.m.)] | 0.00 e | 0.37 c | 0.00 e | 0.39 c | 0.04 d | 0.90 b | 1.51 a |
| Starch, corm [(mg $g^{-1}$(d.m.)] | 1.15 b | 0.57 c | 1.16 b | 1.06 b | 0.59 c | 3.27 a | 3.59 a |
| PK [U $mg^{-1}$(prot.)] | 22.29 d | 284.6 c | 15.43 d | 411.55 b | 34.57 d | 501.25 a | 50.62 d |
| PEPC [U $mg^{-1}$(prot.)] | 6.31 c | 6.33 c | 15.08 a | 10.34 b | 18.51 a | 12.06 b | 17.85 a |

increased by accumulation of reserves, and roots protruded and expanded (Table 2). For the same leaf area (LA), GM favoured the growth of the plantlets with wide and short leaves, whereas TIB gave rise to longer and thinner leaves. Most of the morphological parameters changed mostly during the first 14 d of elongation with two exceptions: the stem diameter which increased in the last week and was not affected by the propagation method; and the number of roots per shoot because roots protruded on day 14 in the TIB and not before day 21 in the GM plantlets.

Photosynthetic and transpiration rates were measured during elongation in plantlets grown under the GM and TIB (Table 2). Until E7, negative values of $CO_2$ uptake were measured, an indicator of respiratory activity. From that time point on, the plantlets were photosynthetically active with a net photosynthetic rate 3- to 4-fold higher in TIB than in GM. Stomatal conductance of the GM grown plantlets increased during the elongation phase, with a significant rise at E14. Conversely, in the TIB grown plantlets, stomatal conductance decreased during elongation, attaining values of one sixth of that in the GM plantlets. However, the transpiration rate in the TIB plantlets was high, certainly due to the constant aeration of the headspace and so much lower air humidity.

The maximum starch accumulation in the corms and leaves was observed on day 21 of elongation, with no differences between GM and TIB in the corms, but higher

values in the leaves of the TIB plantlets as compared to GM (Table 2).

During the elongation period, the PK activity increased steadily in leaves of both the GM and TIB plantlets, although the values were significantly higher in GM (Table 2). The PEPC activity also increased during elongation in both TIB and GM leaves, conversely to PK, PEPC values were significantly higher in the leaves of the TIB plantlets.

Monitoring the expression of the genes encoding PEPC, PK, and starch synthase (SS) during the acclimatization phase (0, 3, 7, and 14 d) in the GM and

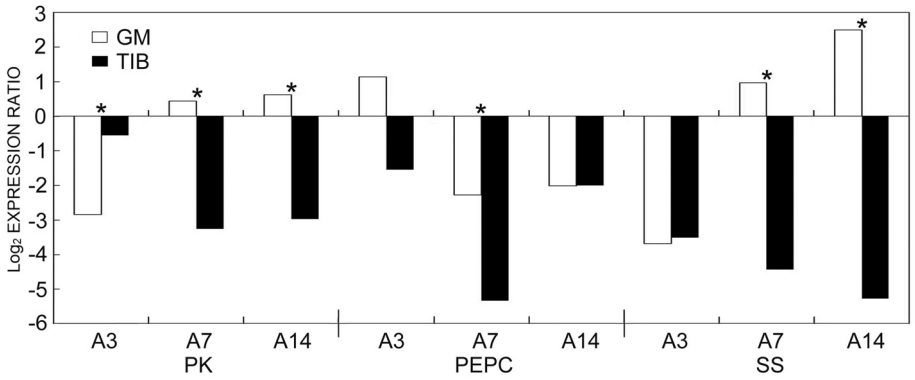


Fig. 1. Patterns of expression of three carbon metabolism genes coding pyruvate kinase (PK), phospho*enol*pyruvate carboxylase ( PEPC), and starch synthase (SS) during acclimatization. Expression was obtained by RT-qPCR monitored during the first 14 d of acclimatization (A3, A7, and A14) after *in vitro* growth in GM and TIB. Expression was normalized to that of *actin 2* and reported 1 at day 0 of acclimatization (* - significant differences at 5 % probability determined by Dunnett multiple range test, $n = 4$). Positive and negative values were processed separately, for negative values, the positive modular equivalent number was used.

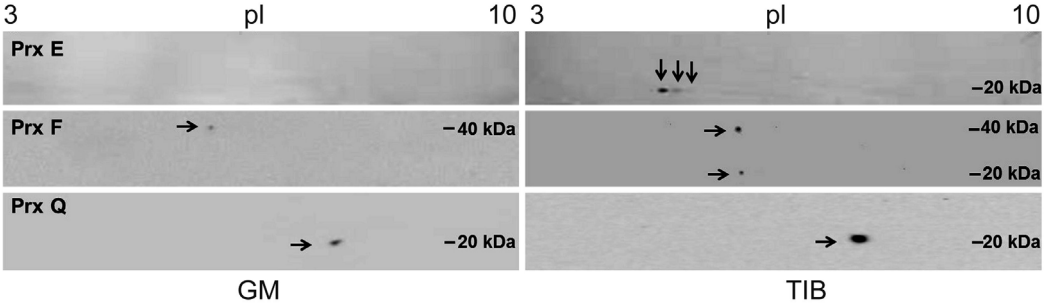


Fig. 2. Comparison of Western blots of three peroxiredoxins (Prx E, F, and Q) in plantain plants propagated in GM and TIB on day 21 of acclimatization. 2-DE gels were transferred to nitro-cellulose membranes and probed with anti-Prx E, F, and Q antibodies, respectively. *Arrows* indicate the isoforms. First dimension, IEF (left to right, pI 3.0 - 10.0); second dimension, SDS-PAGE (top to bottom). Protein markers in kDa are indicated at the side of the gels.

TIB grown plantlets (Fig. 1) can give an insight into reprogramming occurring in carbon metabolism when plantlets endured the stress of transition to *ex vitro* conditions. In GM, *PK* suffered an initial decrease, returning to the basal level after 7 d, whereas in TIB the decrease was steady and values remained low until day 14 (Fig. 1). The trends of *PEPC* expression (Fig. 1) were similar to those in PK in the both types of plantlets except for TIB at the 7$^{th}$ day of acclimatization where a significant down-regulation was observed. In accordance, the TIB grown plantlets also showed a significant decrease in *SS* expression during the whole period of acclimatization, whereas a continuous up-regulation was established in the GM plantlets after the lowest values measured on the third day (Fig. 1).

Total proteins from leaf samples collected on day 21 of acclimatization were separated by 2D electrophoresis and peroxiredoxins (Prxs) E, F, and Q were identified by immunoblotting. TIB showed a larger number and diversity of Prx spots (Fig 2). Three spots corresponding to Prx E were identified, with a molecular mass *ca.* 20 kDa and pI 5 - 6 increasing in visual intensity as pI decreased. In the GM grown plants, Prx F had a spot at pI 5.6, Mr *ca.* 20 kDa, and formed a dimer of 40 kDa. Prx Q showed a spot of high intensity close to pI 5.4 and Mr *ca.* 20 kDa, and spots of lower intensity were also visualized. The Mr and pI were within the expected ones for Prxs in general (Dietz 2003).

The expression of *Prx E, Prx F*, and *Prx Q* genes at the end of acclimatization (21 d) was monitored (Fig. 3). *Prxs E* and *F* were moderately down-regulated in GM, whereas *Prx Q* was strongly up-regulated in the TIB plants. The expression of all *Prxs* monitored was significantly higher in the TIB than in GM grown plants.

## Discussion

The present work aimed to assess the influence of the *in vitro* cultivation method (TIB or GM) on the subsequent *ex vitro* acclimatization of plantain plantlets. The advantages of TIB on plantain morphology during acclimatization were previously described, focusing on physiological parameters and carbon source storage that could be used during the first days of *ex vitro* growth (Aragon *et al*. 2005), and also on the antioxidative response (Aragon *et al.* 2010).

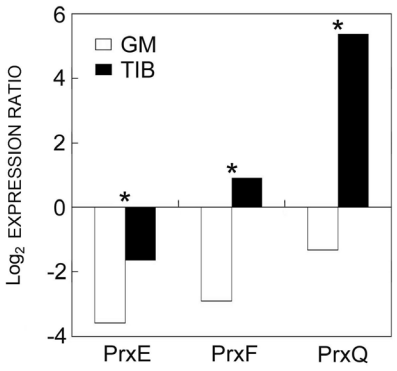


Fig. 3. Quantification of *Prx* gene expression by RT-qPCR (*Prxs E, F, Q*) at the end of acclimatization (A21) of plantain plants previously propagated in GM and TIB. Expression was normalized to that of *actin 2* and is reported 1 at day 0 of acclimatization (* - significant differences between expression levels of each *Prx* in the GM and TIB grown plants at 5 % probability determined by the Dunnett multiple range test, $n = 4$). Positive and negative values were processed separately, for negative values, the positive modular equivalent number was used.

Plants propagated in TIB showed morphological parameters similar to those obtained by Roels *et al*. (2005). They had thinner and longer leaves than the GM plants, a feature similar to naturally grown plantain plants. In fact, the observed differences in growth and also in the physiological parameters between the TIB and GM plantlets can be considered a direct result of the propagation method. In TIB, there is a direct contact of the culture medium with the leaves, leading to the possibility of nutrient uptake by them, what does not happen in GM (Escalona *et al.* 2003, Ziv 2005). This favors the growth of longer leaves that have a higher surface of contact with the medium. During the first period of *in vitro* culture, the plants are more dependent on the nutrients supplied by the culture media than upon autotrophic nutrition (Moreira *et al*. 2003, Larema *et al*. 2012). However, the net photosynthetic rate was higher in the TIB grown plants and the transition from prevailing respiration to photosynthesis was slower in the GM plants. Furthermore, TIB methodology induced a decrease in stomatal conductance, a feature closely related with stomata functioning and regulation of gas exchange. The TIB grown plants have a better regulation of stomatal function and can therefore better control loss of water in the first period of acclimatization, overcoming one of the most frequent causes of plant death at that moment (Hazarika 2003, Perveen *et al*. 2013).

It has been reported previously that the environmental conditions in TIB have a vital influence on growth, physiology, and carbon metabolism of plantain leaves (Aragon *et al*. 2005). These features can certainly explain the significantly larger size and robustness of TIB grown plantlets. One of the main causes for this can be the continuous renewal of the atmosphere surrounding the plantlets, thus allowing the elimination of ethylene in the culture headspace, which was almost undetectable in the TIB vessels, confirming the results previously obtained by Roels *et al*. (2006). Several authors have reported ethylene as a stress-causing compound for plants, impairing normal growth, gas exchange, and normal physiological functioning (Woodrow *et al.* 1988, Outlaw and De Vlieghere-He 2001, Stearns and Glick 2003, Hall *et al*. 2006). It is also possible to assume a higher water use efficiency (net photosynthetic rate/transpiration rate) in the TIB plants. The headspace composition is one of the main contributors for *in vitro* plantlet development (Buddendorf-Joosten and Woltering 1994).

Morphological and physiological changes during the *in vitro* culture were supported by the analysis of carbon metabolism enzymes. The significant increase of PK activity together with the lower PEPC activity (almost 50 fold) in the GM plantlets are in agreement with Le *et al*. (2001) who verified that free access to media sugar sources prevails over the primary synthesis by the plant. In TIB, PEPC activity was higher, possibly due to an environment that mimics outdoor conditions, leading directly to high PEPC activity and photosynthetic rate.

Starch is the main storage compound in plantain plantlets, with a concentration several fold higher in corms than in leaves (Aragón *et al.* 2006). In plantain plantlets propagated in TIB, sucrose is the less important storage form in leaves, further enhancing the importance of starch accumulation and sugar mobilization from corm to leaves in the first days of the *ex vitro* phase (Aragón *et al.* 2006). Leaves can also accumulate starch during the day, the reserves to meet the demands of the plants during night (Capellades *et al.* 1991). It is possible that starch accumulation in the corm depends on an endogenous regulation independent of external factors since in the present work there were no differences between the GM and TIB plantlets. Nevertheless, the starch content in leaves was higher in TIB than in GM due to the higher net photosynthetic rate in the TIB plantlets. During the first days of *ex vitro* growth, plantlets consume their starch reserves, as reported in the other plant species (Capellades *et al.* 1991, Van Huylenbroeck *et al.* 2000, Carvalho *et al.* 2002, Carvalho and Amâncio 2002).

GM plantlets have a higher respiration rate than TIB plantlets, using the sucrose available in the culture media as the basic energy source. The PK activity was 28-fold higher than the PEPC activity in GM, what points to an extremely high catabolic metabolism. The glycolytic

activity supported by the high PK activity compensated the lack of ATP generation by photosynthesis. It is well known that totally closed systems are more stressful than partially closed systems, such as TIB (Ziv 2005).

No significant change in *PK* expression during the transition to autotrophy was observed in the GM plantlets, just a tendency to an initial down-regulation that recovered thereafter whereas in TIB plantlets *PK* was down-regulated. Unlike in GM plantlets, a significant down-regulation of *PEPC* expression in the initial period of acclimatization in the TIB plantlets confirms the stepwise increase of photosynthetic metabolism in *ex vitro* conditions (Aragón *et al*. 2005). The strong down-regulation of *SS* in the TIB plants could be related with the high starch content in leaves of these plantlets. As a whole, the TIB grown plantlets are better adjusted to an autotrophic environment.

Upon exposure to the stress caused by the transfer to *ex vitro* conditions, plantain plants develop an efficient ROS scavenging system (Aragon *et al*. 2010). Among others, Prxs are described as efficient ROS detoxification systems in the chloroplasts, mitochondria and even nucleus, but also as key players in redox signaling during plant development and environmental acclimation (Dietz 2002, 2003). Each Prx has a distinct role and cellular localization. Prx E is present in the chloroplast stroma in very low concentrations, whereas Prxs Q and F have a more prominent role in chloroplasts and mitochondria, respectively (Dietz 2011). In previous research, we showed that polymerization of Prxs E and F is observed in the TIB propagated plants (Aragon *et al*. 2010). A variety of isoforms of Prxs E, F, and Q were clearly identified in TIB plants with higher pI diversity in Prx E and without polymerization patterns in Prx F. Molecular masses for Prx monomers vary between 17.4 and 29.6 kDa and some form dimers, tetramers, and octamers, and their pIs are between 4.71 and 6.29 (Dietz 2003). The results of pI and polymerization of Prxs reflect their cellular compartmentalization between chloroplast and mitochondria. The quantification of the expression of the respective genes show that at the end of acclimatization, *Prxs E* and *F* were down-regulated in GM plants, whereas *Prx Q* was up-regulated in TIB. Even though a direct relationship cannot be established between the gene expression and the enzyme activity in the cell due to the processes taking place between mature RNA formation and enzyme synthesis in the active form, in TIB grown plants the gene expression results mentioned above connect the up-regulation of the expression of *Prx Q* with the respective protein. In unstressed plants, and in plants not being able to respond to a stress, *Prx Q* is only expressed in leaves, but not in roots or stems, and the expression is very low (Rouhier *et al.* 2004). Thus, it is likely that the significant up-regulation of *Prx Q* and the high enzyme activity observed in the TIB grown plants was related to the need to keep hydrogen peroxide concentrations low in order to better overcome the oxidative stress. In fact, the plant oxidative stress defense system during the *in vitro* – *ex vitro* transition was documented before (Carvalho *et al.* 2006, Baťková *et al.* 2008, Dias *et al*. 2013).

In conclusion, the plantlets from the TIB system, which accumulates less ethylene in the headspace, exhibited better growth, lower stomata conductance, and higher photosynthetic rate. Conversely, the GM plants had lower photosynthetic rate and higher PK activity. Further, the TIB grown plantlets accumulated more starch in the leaves which could be used during the first days of the *ex vitro* acclimatization. The occurrence of the different Prx isoforms and the up-regulation of the *Prx Q* expression are further elements that indicate better anti-oxidative performance and thus justify the advantages of the TIB method for producing better quality plants.

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